Binding, Hydration, and Decarboxylation of the Reaction Intermediate Glutaconyl-Coenzyme A by Human Glutaryl-CoA Dehydrogenase†

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ABSTRACT: Glutaconyl-coenzyme A (CoA) is the presumed enzyme-bound intermediate in the oxidative decarboxylation of glutaryl-CoA that is catalyzed by glutaryl-CoA dehydrogenase. We demonstrated glutaconyl-CoA bound to glutaryl-CoA dehydrogenase after anaerobic reduction of the dehydrogenase with glutaryl-CoA. Glutaryl-CoA dehydrogenase also has intrinsic enoyl-CoA hydratase activity, a property of other members of the acyl-CoA dehydrogenase family. The enzyme rapidly hydrates glutaconyl-CoA at pH 7.6 with a k_{cat} of 2.7 s^{-1}. The k_{cat} in the overall oxidation−decarboxylation reaction at pH 7.6 is about 9 s^{-1}. The binding of glutaconyl-CoA was quantitatively assessed from the K_m in the hydratase reaction, 3 μM, and the K_i, 1.0 μM, as a competitive inhibitor of the dehydrogenase. These values compare with K_m and K_i of 4.0 and 12.9 μM, respectively, for crotonyl-CoA. Glu370 is the general base catalyst in the dehydrogenase that abstracts an α-proton of the substrate to initiate the catalytic pathway. The mutant dehydrogenase, Glu370Gln, is inactive in the dehydrogenation and the hydratase reactions. However, this mutant dehydrogenase decarboxylates glutaconyl-CoA to crotonyl-CoA without oxidation−reduction reactions of the dehydrogenase flavin. Addition of glutaconyl-CoA to this mutant dehydrogenase results in a rapid, transient increase in long-wavelength absorbance ($\lambda_{max} \approx 725$ nm), and crotonyl-CoA is found as the sole product. We propose that this 725 nm-absorbing species is the delocalized crotonyl-CoA anion that follows decarboxylation and that the decay is the result of slow protonation of the anion in the absence of the general acid catalyst, Glu370(H^+). In the absence of detectable oxidation−reduction, the data indicate that oxidation−reduction of the dehydrogenase flavin is not essential for decarboxylation of glutaconyl-CoA.

Glutaryl-CoA[1] dehydrogenase is a homotetrameric flavoprotein dehydrogenase (α_4) that catalyzes the oxidation of glutaryl-CoA to crotonyl-CoA, CO_2, and 2 equiv of a one-electron-reduced electron acceptor such as the physiological electron acceptor, electron-transfer flavoprotein (ETF), or a suitable artificial electron acceptor (*1, 2*). The reaction occurs in three discrete steps, and the mechanism of the reductive half-reaction (reaction 1) is very similar to that of other members of the acyl-CoA dehydrogenase family (*3*−*5*).

$$E{\cdot}FAD_{ox} + \text{glutaryl-CoA} \rightleftharpoons [E{\cdot}FADH_2{\cdot}\text{glutaconyl-CoA}] \quad (1)$$

$$[E{\cdot}FADH_2{\cdot}\text{glutaconyl-CoA}] + ETF_{ox} \rightleftharpoons [E{\cdot}FAD_{1e^-}{\cdot}\text{crotonyl-CoA}] + CO_2 + ETF_{1e^-} \quad (2)$$

$$[E{\cdot}FAD_{1e^-}{\cdot}\text{crotonyl-CoA}] + ETF_{ox} \rightleftharpoons E{\cdot}FAD_{ox} + \text{crotonyl-CoA} + ETF_{1e^-} \quad (3)$$

The reductive half-reaction of the dehydrogenase flavin (reaction 1) involves abstraction of the *pro-R*-hydrogen at C-2 of the substrate by Glu370, the catalytic base, followed by or concerted with hydride transfer from C-3 to the flavin prosthetic group (*3*−*5*). The *trans*-2-enoyl-CoA product of the reductive half-reaction is presumed to be glutaconyl-CoA; however, this intermediate has never been directly demonstrated. Crotonyl-CoA and CO_2 are the sole products derived from glutaryl-CoA and it is assumed that glutaconyl-CoA remains tightly bound to the enzyme in the catalytic pathway (*1, 2*). Gomes et al. (*6*) showed that reoxidation of the flavin in *Pseudomonas fluorescens* glutaryl-CoA dehydrogenase was required for decarboxylation when the enzyme was reduced with glutaryl-CoA. Because crotonyl-CoA is capable of stabilizing the semiquinone form of the dehydrogenase flavin in the *Paracoccus denitrificans* dehydrogenase, Byron et al. (*7*) proposed that one-electron oxidation of the flavin dihydroquinone (reaction 2) is necessary for decarboxylation of glutaconyl-CoA.

Chemical synthesis of glutaconyl-CoA is difficult. However, Bückel and co-workers (*8, 9*) reported the enzymatic synthesis of glutaconyl-CoA from glutaconate and acetyl-CoA using a recombinant glutaconate-CoA transferase from *Acidaminococcus fermentans*. This strategy permitted the synthesis of glutaconyl-CoA in good yield to investigate the

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[1] Abbreviations: CoA, coenzyme A; ETF, electron-transfer flavoprotein; FAD, flavin adenine dinucleotide; GCD, glutaryl-CoA dehydrogenase; $FcPF_6$, ferrocenium hexafluorophosphate.

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interaction of glutaryl-CoA dehydrogenase with the intermediate and decarboxylation of this 2-enoyl-CoA product of the reductive half-reaction of the dehydrogenase with glutaryl-CoA. Hydration of the presumed intermediate by an intrinsic enoyl-CoA hydratase activity of human glutaryl-CoA dehydrogenase was also investigated. Hydration of glutaconyl-CoA requires the catalytic base, Glu370. The wild-type dehydrogenase and the Glu370Gln and Arg94Gln mutants catalyze decarboxylation of glutaconyl-CoA without oxidation−reduction reactions of the flavin. In the experiments with the Glu370Gln mutant, a long-wavelength-absorbing (725 nm) species rapidly appears and then decays with first-order kinetics, suggesting that it may be the delocalized anion of crotonyl-CoA.

EXPERIMENTAL PROCEDURES

Materials. Ferrocenium hexafluorophosphate ($FcPF_6$) was obtained from Aldrich. Glutaconic acid (≥97%) was purchased from Fluka and used without further purification. Acetyl-CoA, glutaryl-CoA, CoASH, and crotonyl-CoA were purchased from Sigma. Alternatively, glutaryl-CoA, crotonyl-CoA, and acetyl-CoA were synthesized by reaction of the corresponding anhydrides with CoASH and purified as described (*5*, *10*). 3-Hydroxyglutaryl-CoA was synthesized enzymatically from glutaconyl-CoA by use of purified porcine heart crotonase (2720 units/mg), which was the generous gift of Dr. Horst Schulz, City College of New York (*11*). 3-Hydroxybutyryl-CoA was synthesized enzymatically from crotonyl-CoA by use of crotonase purchased from Sigma.

Enzymes. Wild-type recombinant human glutaryl-CoA dehydrogenase and the mutant glutaryl-CoA dehydrogenases, Glu370Gln and Arg94Gln, were expressed and purified as described by Dwyer et al. (*5*, *12*).

Synthesis of Glutaconyl-CoA. The plasmid pMM2, containing the two genes encoding the *Acidaminococcus fermentans* glutaconate coenzyme A-transferase subunits, was a generous gift from Dr. Wolfgang Bückel (*9*). Recombinant glutaconate coenzyme A-transferase was expressed and purified from *Escherichia coli* DH5α (*9*). The specific activity of the transferase preparations used in this study varied from 132 to 137 units/mg.

The synthesis of glutaconyl-CoA from acetyl-CoA and glutaconate was carried out as described (*13*), on a 30 mL scale with 400 units of the partially purified CoA transferase. The synthesis was carried out at room temperature and followed spectrophotometrically at 280 nm in a 0.1 cm path length quartz cuvette. Equilibrium was reached in 25−30 min. The pH of the reaction was adjusted to 4−5 with 0.5 M H_2SO_4 and denatured protein was removed by centrifugation. The reaction was diluted 5-fold with H_2O and applied to a DEAE-cellulose column (2.5 × 15 cm) equilibrated with 3 mM HCl and washed with 4 column volumes of 3 mM HCl. The column was then eluted with a linear gradient (1400 mL) to 0.25 M LiCl in 3 mM HCl. This chromatography separated unreacted glutaconate and acetyl-CoA from glutaconyl-CoA. The product, glutaconyl-CoA, was desalted by chromatography on Sephadex G-10. Glutaconyl-CoA was lyophilized and stored dry at −80 °C.

The purity of glutaconyl-CoA was assessed by mass spectrometry and analytical HPLC. Mass spectrometry of the synthetic reaction product showed a single compound ($[M + H]^+$, *m*/*z* 880.6) as expected. The purified product was shown to have a ratio A_{260nm}/A_{232nm} of 1.65−1.69, which compares to a ratio of 1.7 for crotonyl-CoA and a ratio of about 2.0 for saturated acyl-CoAs. All of these data were consistent with the expected structure. However, by the gradient HPLC method (*14*), a second peak that eluted about 2 min after the main peak of glutaconyl-CoA was detected. This latter peak consistently contributed 15−20% of the total glutaconyl-CoA. The minor component reappeared when the main peak of glutaconyl-CoA was rechromatographed in solvent system B (*14*). ^1H NMR spectroscopy indicated the presence of about 15% *trans*-3-glutaconyl-CoA, which is consistent with the observation that the transferase exhibits some isomerase activity to yield the resonance-stabilized *trans*-3-isomer (*13*).

Analytical Methods. $Na_2S_2O_4$ was standardized by anaerobic titration of $K_3Fe(CN)_6$ (*15*). Acyl-CoA esters for analysis by HPLC and mass spectrometry were isolated from reaction mixtures essentially as described by Corkey (*14*). Trichloroacetic acid was added to a final concentration of 10%, and precipitated protein was removed by centrifugation. The trichloracetic acid was removed by five extractions with an equal volume of diethyl ether, and the resulting aqueous layer was lyophilized. The sample was taken up in a minimal amount of H_2O and adjusted to pH 3.0 with 0.1 mL of 0.5 M phosphoric acid. The reaction mixture was applied to a C_{18} Sep-Pak cartridge, which was then washed extensively with H_2O, pH 3.0, and the acyl-CoA esters were eluted with methanol. Samples were dried, resuspended in 50 μL of water, and analyzed by mass spectrometry and analytical reversed-phase HPLC in the acetonitrile/potassium phosphate gradient (*14*). Alternatively, some incubations were conducted in 0.01 M $(NH_4)CO_2H$, pH 7.6, and acidified with acetic acid to pH 3.0, and protein was removed with an Amicon Centricon YM30 centifugal filter. When the enzyme-bound acyl-CoAs were determined after anaerobic reduction with substrate, we used a modification of the rapid quench method of Lau and Thorpe (*16*). The reaction was terminated with an equal volume of anaerobic 20% trichloroacetic acid, which was substituted for 2 M HCl in the original procedure. Samples were processed as described above for the trichloroacetic acid precipitation protocol. Acyl-CoA thioesters were analyzed by mass spectrometry and HPLC in solvent system A (*13*), and/or solvent system B (*14*) on a 5 μm, C_{18} reverse-phase column (0.45 × 25 cm) as described.

Analytical HPLC was conducted with the following solvent systems. In solvent system A, the column was developed isocratically with 85% 0.05 M potassium phosphate, pH 2.5/15% methanol (*13*). Solvent system B was a gradient of 0.1 M potassium phosphate, pH 5.0/acetonitrile described by Corkey (*14*). Retention times (in minutes) of pertinent analytes in solvent system B were as follows: CoASH (37), glutaconyl-CoA (59), glutaconyl-CoA *trans*-3-isomer (61), acetyl-CoA (60), glutaryl-CoA (61), 3-hydroxyglutaryl-CoA (62), 3-hydroxybutyryl-CoA (62), FAD (64), and crotonyl-CoA (67). Acyl-CoA standards were chromatographed immediately before a sample was analyzed to ensure accuracy of retention times. Elution was monitored at 260 nm and relative concentrations were determined by integration of the area under the peaks.

Since the elution times of some acyl-CoA esters were very similar, samples were also analyzed by mass spectrometry. Electrospray ionization mass spectra were obtained with a PE Sciex API-3000 triple-quadrupole mass spectrometer with a turbo ionspray source, interfaced with a Perkin-Elmer Series 200 HPLC system. Samples were analyzed in flow injection mode with a Phenomenex C_{18} guard column in isocratic 75% methanol in water containing 0.01 M ammonium acetate at a flow rate of 200 μL/min and sample injection volume of 20 μL. The mass spectrometer settings were as follows: turbo ionspray temperature, 300 °C; needle spray, 4500 V; declustering potential, 30 V; focus plate, 175 V. Mass spectra were acquired from Q1 in the positive-ion mode, scanning the mass range from 500 to 1200 amu every 3 s.

^{1}H NMR spectra were acquired with a Bruker Avance DRX 500 operating at 500.13 MHz.

Anaerobic experiments were carried out after repeated evacuation and purging of the reaction mixtures with argon. The reactions contained 0.4 mM protocatechuate and, after evacuation and purging, were treated with 0.5 unit of protocatechuate dioxygenase to remove residual oxygen (*17*).

Enzyme Assays. Glutaryl-CoA dehydrogenase was routinely measured in reactions containing 0.01 M potassium phosphate, pH 7.6, 30 μM glutaryl-CoA, and 200 μM $FcPF_6$ ($\epsilon_{300nm} = 4.3 \times 10^3$ M^{-1} cm^{-1}) as the terminal electron acceptor (*5*). When glutaconyl-CoA and crotonyl-CoA were evaluated as inhibitors of GCD with respect to glutaryl-CoA, the data were fit to the following equation for competitive inhibition by Grafit 4.0 (Erithacus Software Ltd.):

$$v = \frac{V_{max}[S]}{K_m\left(1 + \frac{[I]}{K_i}\right) + [S]}$$

Enoyl-CoA hydratase activity was determined spectrophotometrically by following the decrease in absorbance at 280 nm due to the hydration of the *trans*-2 double bonds of glutaconyl-CoA or crotonyl-CoA. Reaction mixtures contained 0.05 M potassium phosphate buffer, pH 7.0, and 50 μM enoyl-CoA unless otherwise indicated. Activity was calculated by using $\Delta\epsilon_{280nm} = 3.6 \times 10^3$ M^{-1} cm^{-1} (*18*). The glutaconate coenzyme A-transferase was assayed with glutaryl-CoA and acetate as substrates; acetyl-CoA formation was determined in the coupled assay described by Bückel et al. (*8*).

RESULTS

Products of Glutaryl-CoA Oxidation. Glutaryl-CoA (30 μM) was incubated aerobically with 40 nM glutaryl-CoA dehydrogenase and 200 μM $FcPF_6$ as the electron acceptor in 0.05 M potassium phosphate, pH 7.6, at 25 °C. The reaction was followed to completion by monitoring the decrease of absorbance at 300 nm due to reduction of $FcPF_6$. The product of the reaction was crotonyl-CoA, as determined by HPLC (elution time = 67 min) and mass spectrometry ($[M + H]^+$, *m*/*z* 836.5, not shown). These results (Figure 1) are consistent with the experiments of Lenich and Goodman (*1*) and Besrat et al. (*2*).

Since these early experiments (*1*, *2*), the working hypothesis for the mechanism of glutaryl-CoA has been that

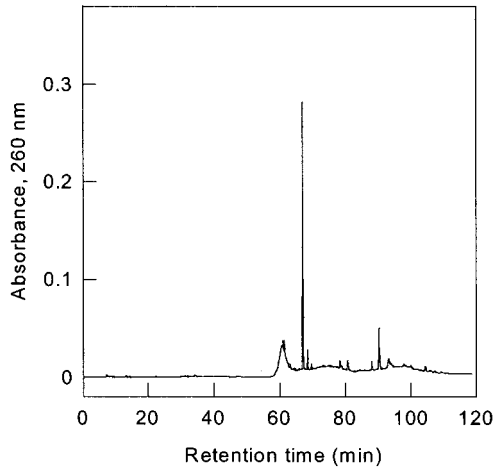

FIGURE 1: Identification of the acyl-CoA product of glutaryl-CoA dehydrogenase turnover by HPLC. Glutaryl-CoA (30 μM) was oxidized with 40 nM GCD in the presence of 200 μM $FcPF_6$ as the electron acceptor in 0.05 M potassium phosphate, pH 7.6, 25 °C. The reaction was followed to completion by monitoring the decrease in absorbance at 300 nm due to reduction of $FcPF_6$. The product, crotonyl-CoA, was identified by HPLC in solvent system B (retention time 67 min) and by mass spectrometry ($[M + H]^+$ *m*/*z* 836.5).

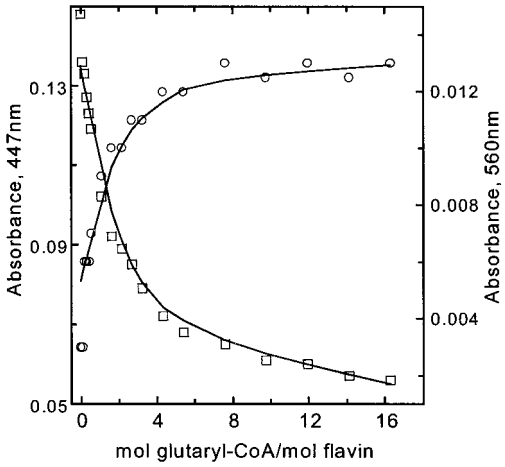

FIGURE 2: Anaerobic titration of wild-type GCD with glutaryl-CoA. The dehydrogenase (9.2 μM) was titrated with glutaryl-CoA under anaerobic conditions in 0.05 M potassium phosphate, pH 7.6, and 10% glycerol, 25 °C. Complete spectra were recorded, and the figure shows the changes in the absorbance at 447 nm (□), the visible absorption maximum of the flavin, and at 560 nm (○), the absorption maximum of the charge transfer complex, as a function of the glutaryl-CoA:flavin ratio. The lines represent a locally weighted least-squares fit that yields the best smooth curves through the experimental data points.

glutaconyl-CoA is generated in the reductive half-reaction of the dehydrogenase flavin (reaction 1). Gomes et al. (*6*) proposed that glutaconyl-CoA remains enzyme-bound and is decarboxylated when the dehydrogenase flavin is reoxidized by electron-transfer flavoprotein or a suitable artificial electron acceptor (reactions 2 and 3).

In a previous paper, we showed that in an anaerobic titration of wild-type GCD with glutaryl-CoA, equimolar substrate reduced only 23−26% of the flavin (*5*). Figure 2 shows the results of a similar experiment and illustrates the extent of reduction of the flavin and the formation of the charge-transfer species as a function of the substrate/flavin ratio. The redox poise of the system is much different from that of medium-chain acyl-CoA dehydrogenase, in which

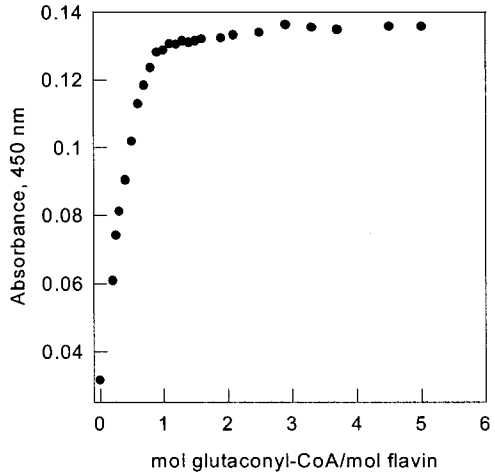

FIGURE 3: Anaerobic titration of dithionite-reduced GCD with glutaconyl-CoA. Wild-type GCD (15 μM) in 0.05 M potassium phosphate, pH 7.6, 25 °C, was reduced by careful titration with $Na_2S_2O_4$ that was standardized by anaerobic titration of $K_3Fe(CN)_6$. The fully reduced enzyme was then titrated with an anaerobic solution of glutaconyl-CoA. The figure shows the absorbance at 447 nm as a function of the glutaconyl-CoA:flavin ratio.

equimolar substrate reduces about 80% of the flavin (*19*). Figure 3 shows the flavin absorbance as a function of the glutaconyl-CoA/flavin ratio in an anaerobic titration of the fully reduced dehydrogenase with glutaconyl-CoA. The dehydrogenase was reduced with equimolar $Na_2S_2O_4$. The flavin is reoxidized by stoichiometric glutaconyl-CoA. There is no anionic or neutral semiquinone detected during the reductive titration (*5*) or the back-titration with glutaconyl-CoA. When the reduced enzyme was back-titrated with crotonyl-CoA, the results were essentially identical (not shown). The experiments described in Figures 2 and 3 demonstrate the interplay of (a) equilibria involved in glutaryl-CoA binding to the oxidized enzyme and internal redox equilibria and (b) the binding of glutaconyl-CoA to the reduced enzyme and reversed electron transfer that reflects how the enoyl-CoA modulates flavin potential in the physiological reaction (*5*).

In the experiments shown in Figure 4, we assessed the redox equilibrium at the active site. In these experiments, glutaryl-CoA was added to the enzyme in a 10% molar excess over flavin, and the incubation was rapidly quenched with an equal volume of anaerobic 20% trichloroacetic acid. The precipitated dehydrogenase was removed by centrifugation. Acyl-CoAs were determined by HPLC and mass spectrometry (Figure 4). The chromatogram (Figure 4A) shows glutaconyl-CoA (59 min), the *trans*-3-isomer of glutaconyl-CoA (61 min) and/or glutaryl-CoA (61 min), FAD (64 min), and crotonyl-CoA (67 min). The ratio of glutaryl-CoA:glutaconyl-CoA was (2.5 ± 0.2):1 in three experiments. This result is in reasonable agreement with the level of reduction of GCD flavin by equimolar glutaryl-CoA under anaerobic conditions (Figure 2 and ref 5). There is a possibility that the low pH in the acid denaturation shifts internal redox equilibria that may be more than denaturation; however, our results do reflect the level of reduction of GCD by glutaryl-CoA. The *trans*-3-isomer of glutaconyl-CoA, glutaryl-CoA, 3-hydroxyglutaryl-CoA, and 3-hydroxybutyryl-CoA coelute on HPLC; therefore, the products of the reaction were also identified by mass spectrometry. Mass spectrometry of the products of the reaction (Figure 4B)

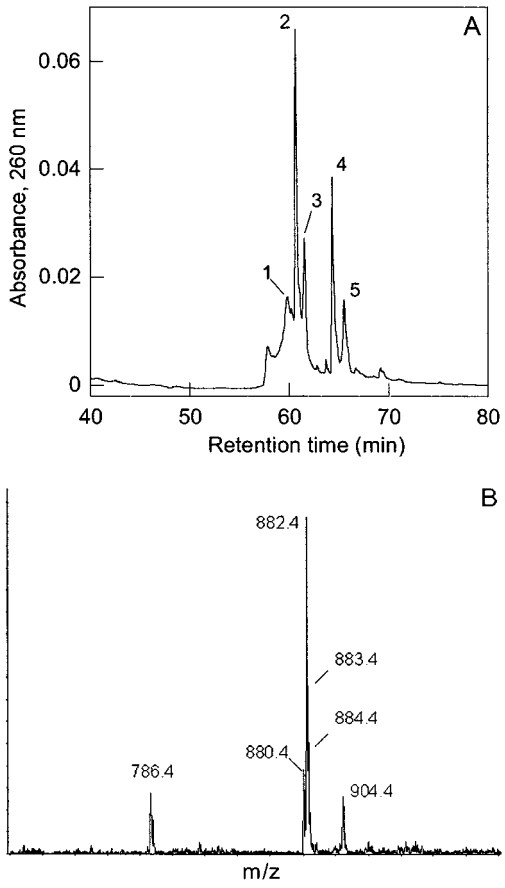

FIGURE 4: Analysis of the acyl-CoA thioesters released from GCD reduced with a 10% molar excess of glutaryl-CoA after acid denaturation. GCD (30 μM) was reduced with a 10% molar excess of glutaryl-CoA under anaerobic conditions. The incubation was rapidly quenched with anaerobic 10% trichloroacetic acid. The processing and chromatography was as described under Experimental Procedures. The products of the reaction were analyzed by HPLC (panel A) with solvent system B. The identities of the analytes, based on retention times (in minutes), are as follows: [1] glutaconyl-CoA (59); [2] glutaryl-CoA and/or the *trans*-3-isomer of glutaconyl-CoA (61); [3] 3-hydroxyglutaryl-CoA (62); [4] FAD (64); [5] crotonyl-CoA (67). Panel B shows the mass spectrum of the acid-soluble fraction after rapid quench and processing. $[M + H]^+$, *m/z* 786.4, FAD; $[M + H]^+$, *m/z* 880.4, glutaconyl-CoA; $[M + H]^+$, *m/z* 882.4, glutaryl-CoA; $[M + Na]^+$, *m/z* 904.4, sodium glutaryl-CoA. The peak *m/z* 883.4 is a mixture of ^{13}C, ^{2}H, and ^{15}N isotopes. The peak *m/z* 884.4 is a mixture of ^{13}C and ^{18}O isotopes.

shows glutaconyl-CoA ($[M + H]^+$, *m/z* 880.4), and glutaryl-CoA ($[M + H]^+$, *m/z* 882.4). The amount of crotonyl-CoA detected by HPLC was variable and was not usually detected by mass spectrometry ($[M + H]^+$, *m/z* 836.6). The occasional presence of crotonyl-CoA may be due to some O_2 when the reaction was quenched and the oxidase activity of GCD. Crotonyl-CoA was not detected in the mass spectrum of the experiment shown in Figure 4. Other species detected in the mass spectrum (Figure 4B) are FAD ($[M + H]^+$, *m/z* 786.4) and the sodium salt of glutaryl-CoA ($[M + Z]^+$, *m/z* 904.4).

Binding and Hydration of Glutaconyl-CoA by Glutaryl-CoA Dehydrogenase. 3-Hydroxyglutaryl-CoA was occasionally observed in experiments such as that shown in Figure 4. The presence of 3-hydroxyglutaryl-CoA suggested that, like *Paracoccus* GCD, *Megasphera elsdenii* short-chain acyl-CoA dehydrogenase and medium-chain acyl-CoA dehydrogenase (*7, 20, 21*), oxidized human wild-type GCD has intrinsic enoyl-CoA hydratase activity and that glutaconyl-

Table 1: Steady-State Kinetic Constants for Glutaconyl-CoA and Crotonyl-CoA as Substrates of the Enoyl-CoA Hydratase Activity of Glutaryl-CoA Dehydrogenase and as Competitive Inhibitors with Respect to Glutaryl-CoA of the Dehydrogenation Activity of Human Glutaryl-CoA Dehydrogenase

	enoyl CoA hydratase		dehydrogenase
enzyme	substrate	activity[a] (s^{-1})	K_i[b] (μM)
wild-type GCD	glutaconyl-CoA	2.7 ± 0.2[c]	1.1 ± 0.3
	crotonyl-CoA	0.17 ± 0.01[c]	12.8 ± 1.6
Arg94Gln	glutaconyl-CoA	0.08	
	crotonyl-CoA	0.01	
Glu370Gln	glutaconyl-CoA	≤0.003	
	crotonyl-CoA	≤0.003	

[a] Specific activity expressed as turnover with 50 mM glutaconyl-CoA or crotonyl-CoA for the two mutants. [b] K_i for gluatconyl-CoA and crotonyl-CoA as competitive inhibitors of glutaryl-CoA dehydrogenase with respect to glutaryl-CoA. [c] kcat from extrapolation to infinite glutaconyl-CoA or crotonyl-CoA concentration; Kmglutaconyl-CoA = 3.0 ± 0.7 mM; $K_m^{\text{crotonyl-CoA}} = 23.1 \pm 5.2$ μM.

CoA could be hydrated by the dehydrogenase. Quantitation of the hydratase activity of wild-type GCD and the Glu370Gln and Arg94Gln mutant dehydrogenases with glutaconyl-CoA and crotonyl-CoA as substrates are given in Table 1. The hydratase activities of the mutant enzymes are specific activities expressed as apparent turnover numbers. The activity of wild-type GCD with glutaconyl-CoA is a true turnover number, 2.7 ± 0.2 s^{-1}, and the K_m of glutaconyl-CoA with wild-type GCD in the hydratase reaction is 3.0 ± 0.7 μM. With crotonyl-CoA as substrate, k_{cat} with wild-type GCD is 0.17 ± 0.01 s^{-1}, and the K_m of crotonyl CoA is 23.1 ± 5.2 μM. The difference in free energy of binding of glutaconyl-CoA and crotonyl-CoA estimated from $\Delta\Delta G_b = -RT \ln (K_m^{\text{glutaconyl-CoA}}/K_m^{\text{crotonyl-CoA}})$ is 5.1 kJ/mol (*22*), about equal to the energy of formation of a hydrogen bond. The k_{cat} of the overall oxidation-decarboxylation reaction catalyzed by GCD at pH 7.6 is about 9 s^{-1}. The hydratase activity of the reduced enzymes could not be determined because addition of glutaconyl-CoA to a reduced enzyme simply reoxidizes the free enzyme.

The Glu370Gln mutation abolishes detectable hydratase activity with glutaconyl-CoA and crotonyl-CoA, and the activity of Arg94Gln GCD is reduced 97% with glutaconyl-CoA and 75% with crotonyl-CoA as the substrates. The percent decrease in k_{cat} with glutaconyl-CoA is identical to the percent decrease in k_{cat} for the dehydrogenation of glutaryl-CoA (*12*). The basis for the decrease in activity with crotonyl-CoA as substrate is not known.

Wild-type GCD hydrates glutaconyl-CoA; thus it was not possible to determine the binding constant by spectrophotometric titration. However, the K_m for glutaconyl-CoA in the hydratase reaction provides some measure of the binding of glutaconyl-CoA and a comparison of binding with crotonyl-CoA, which dissociates from the protein in the normal catalytic pathway. To pursue this point further, we determined the inhibition of GCD by glutaconyl-CoA and crotonyl-CoA with glutaryl-CoA as the varied substrate. These results are also shown in Table 1. The kinetic K_i, 1.1 ± 0.3 μM, of glutaconyl-CoA as a competitive inhibitor with respect to glutaryl-CoA is similar to the K_m, 3.0 ± 0.7 μM, for glutaconyl-CoA in the hydratase reaction, and these values are about 7−10-fold less than the K_i, 12.9 ± 1.6 μM, for crotonyl-CoA as a competitive inhibitor with respect to glutaryl-CoA. Estimating the difference in the free energy of binding from the inhibitor data yields a $\Delta\Delta G_b = 6$ kJ/mol, essentially identical to the value calculated from K_m values, 5 kJ/mol (*22*). These results are consistent with the role of glutaconyl-CoA as a bound intermediate that is rapidly decarboxylated and indicates that a relatively small $\Delta\Delta G_b$ maintains glutaconyl-CoA in a bound state for subsequent decarboxylation.

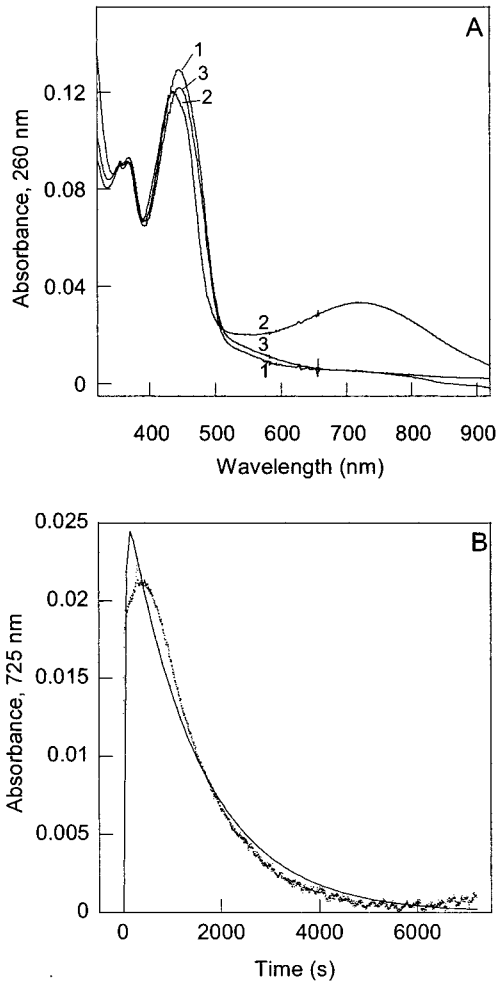

FIGURE 5: Spectral changes observed upon addition of glutaconyl-CoA to Glu370Gln glutaryl-CoA dehydrogenase. (A) Oxidized dehydrogenase, 15 μM, in 0.01 M ammonium formate, pH 7.6, 25 °C (1); the dehydrogenase immediately after addition of 28 μM glutaconyl-CoA (2); and the absorption spectrum of the enzyme and glutaconyl-CoA mixture 30 min after addition of glutaconyl-CoA (3). (B) Rate of formation and decay of the 725 nm-absorbing species; the line is a fit of the data to sequential first-order reactions ($R^2 = 0.96$). The reaction was carried out at 25 °C in 0.05 M potassium phosphate, pH 7.5, containing 5% ethylene glycol; the concentration of enzyme was 10 μM and the concentration of glutaconyl-CoA was 25 μM.

Decarboxylation of Glutaconyl-CoA. Glu370Gln GCD does not hydrate glutaconyl-CoA, but it was not possible to estimate a K_d for glutaconyl-CoA binding by direct spectrophotometric titration because it became evident that a further conversion of glutaconyl-CoA was catalyzed by Glu370Gln GCD. Figure 5A shows the spectra of free Glu370Gln GCD, 15 μM, and the binary complex with glutaconyl-CoA immediately after mixing with 30 μM glutaconyl-CoA. The absorption maximum at 444 nm is blue-shifted by 5 nm and there is a striking increase in long-wavelength absorbance with a maximum at about 725 nm. The chemical identity of

this species was not immediately obvious, but the absorption spectrum is similar to those of acyl-CoA dehydrogenase binary complexes with acyl-CoA substrate analogues such as acetoacetyl-CoA, 3-oxoacyl-CoA, and 3-thiaacyl-CoA thioesters. These nonoxidizable acyl-CoA analogues form charge-transfer species of the oxidized flavin with the delocalized C-2 anions (*5, 23*−*25*). A transient long-wavelength-absorbing species ($\lambda_{max} \approx 800$ nm) is also formed during the inactivation of short-chain acyl-CoA dehydrogenase by 2-pentynoyl-CoA (*26*). The latter species is presumably the delocalized anion that forms after deprotonation at C-4, which then decays upon formation of the allene and covalent modification of the catalytic glutamate in the active site. However, as shown in Figure 5, the absorbance at 725 nm is not stable. The absorption maximum of the flavin in the 450 nm region shifts back to the red. Figure 5B shows the increase in absorbance at 725 nm and the subsequent decrease in absorbance. The curve is fitted to two sequential first-order reactions with apparent rate constants of 0.04 min^{-1} for the formation of the 725 nm-absorbing species (k_1) and 1.5 min^{-1} for the decay (k_2) at pH 7.5. The pH dependence was determined for both processes between pH 6.5 and 8.0, where the mutant enzyme is most stable. The values of k_1 at pH 6.5, 7.0, 7.5, and 8.0 are 0.04, 0.05, 0.04, and 0.03 min^{-1}, respectively, and the values of k_2 at the same pH values are 1.2, 2.5, 1.5, and 1.1 min^{-1}, respectively. The standard error for each rate constant is less than 1% and the correlation coefficients, R^2, are greater than 0.96.

The product of this reaction at pH 7.6 (Figure 5A) was analyzed by mass spectrometry and HPLC in solvent system B after 10 min and after 30 min. After 10 min, 75% of the glutaconyl-CoA was converted to crotonyl-CoA (Figure 6A), and after 30 min, 95% of the glutaconyl-CoA was converted to crotonyl-CoA (data not shown). Crotonyl-CoA was also identified by mass spectrometry ($[M + H]^+$ *m/z* 836.4, Figure 6B). Compound 1 has the same retention time as *trans*-3-glutaconyl-CoA, glutaryl-CoA, 3-hydroxyglutaryl-CoA, and 3-hydroxybutyryl-CoA. However, none of these compounds was detected by mass spectrometry, so that compound 1 has not been identified. Addition of crotonyl-CoA or 2-pentenoyl-CoA to Glu370Gln GCD did not produce the 725 nm-absorbing species, although addition of 2-pentenoyl-CoA to the oxidized mutant enzyme resulted in an 8% decrease in absorbance at 447 nm, and a 9 nm red shift of the absorption maximum to 456 nm. This is similar to the spectrum of the binary complex between medium-chain acyl-CoA dehydrogenase and 2-octenoyl-CoA (*27*).

We also investigated the reaction of oxidized wild-type and Arg94Gln GCD with glutaconyl-CoA. When glutaconyl-CoA was added to these forms of GCD, the long-wavelength-absorbing species that was observed with Glu370Gln GCD was not detected (data not shown). However, after a 10 min incubation of glutaconyl-CoA with wild-type GCD, the reaction was quenched and approximately 99% of the product was identified by HPLC and mass spectrometry as 3-hydroxybutyryl-CoA ($[M + H]^+$, *m/z* 854.4) (Figure 7). These data indicate that the wild-type protein can decarboxylate substrate in its oxidized form, and it is likely that hydration precedes decarboxylation. In the case of Arg94Gln GCD, the products detected by HPLC and mass spectrometry included mostly crotonyl-CoA and some 3-hydroxyglutaryl-CoA (Figure 8).

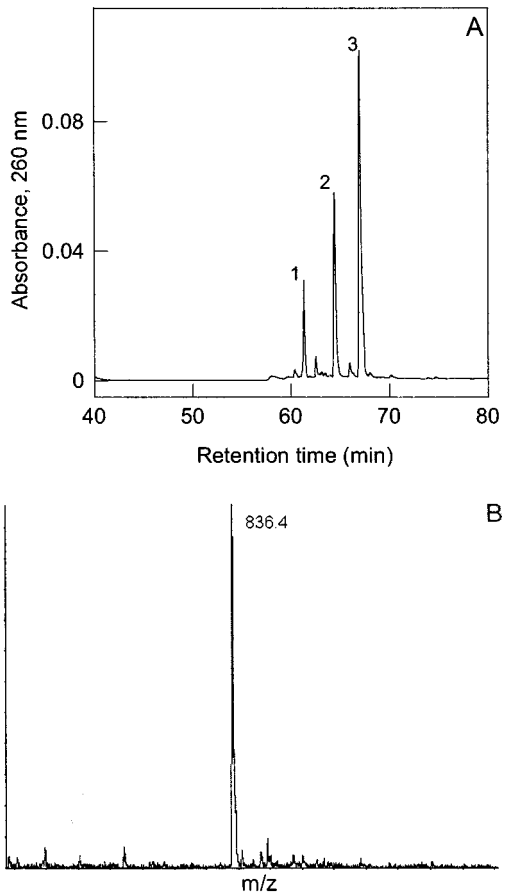

FIGURE 6: Identification of the product generated in an incubation of glutaconyl-CoA with Glu370Gln glutaryl-CoA dehydrogenase. The mutant dehydrogenase, 10 μM, in 0.01 M ammonium formate, pH 7.6, 25 °C, was incubated with 28 μM glutaconyl-CoA for 10 min. The reaction was quenched by adjusting the pH to 3.0 with 1 M acetic acid and the enzyme was removed by centrifugal filtration. (A) HPLC chromatogram of the reaction mixture in solvent system B; the compounds were identified by retention times (in minutes): [1] *trans*-3-isomer of glutaconyl-CoA (61); [2] FAD (64); [3] crotonyl-CoA (67). (B) Mass spectrum of the analytes in the reaction mixture identified crotonyl-CoA ($[M + H]^+$, *m/z* 836.4).

DISCUSSION

The acyl-CoA product of the overall reaction catalyzed by glutaryl-CoA dehydrogenase is crotonyl-CoA. In the first experiment, we demonstrated the production of crotonyl-CoA under steady-state conditions from the turnover of GCD in the presence of an electron acceptor. This experiment serves as a baseline and agrees with the work of others (*1,2*). However, unlike previous studies with mammalian and bacterial glutaryl-CoA dehydrohenases (*1, 2, 6, 7*), we demonstrated glutaconyl-CoA as an enzyme-bound intermediate when the GCD flavin is reduced with equimolar glutaryl-CoA in the reductive half-reaction under anaerobic conditions. This intermediate has been proposed but never demonstrated (*1,7*). Lau et al. (*21*) showed that upon addition of equimolar octanoyl-CoA to medium chain acyl-CoA dehydrogenase under anaerobic conditions, 2-octenoyl-CoA and octanoyl-CoA were recovered in a 9:1 ratio. In the present work, glutaconyl-CoA and glutaryl-CoA were recovered in a 1:2.5 ± 0.2 ratio when glutaryl-CoA was added to wild-type GCD under anaerobic conditions. This result is consistent with the extent of flavin reduction in the anaerobic titration of the wild-type enzyme with glutaryl-CoA, and is

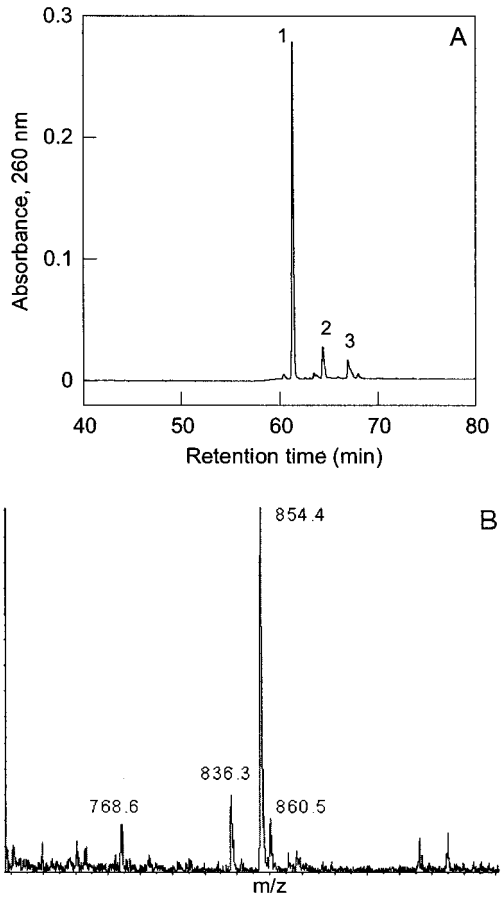

FIGURE 7: Identification of the product generated by incubation of glutaconyl-CoA with wild-type GCD. The dehydrogenase, 10 μM, in 0.01 M ammonium formate, pH 7.6, 25 °C, was incubated with 28 μM glutaconyl-CoA for 10 min. The reaction was quenched by adjusting the pH to 3.0 with 1 M acetic acid, and the enzyme was removed by centrifugal filtration. (A) HPLC of the filtrate in solvent system B identified the compounds by retention times (in minutes): [1] *trans*-3-isomer of glutaconyl-CoA (61) and/or 3-hydroxyglutaryl-CoA (62) and/or 3-hydroxybutyryl-CoA (62); [2] FAD (64); [3] crotonyl-CoA (67). (B) Mass spectrum of the analytes of the reaction mixture identified coenzyme A ($[M + H]^+$, *m/z* 768.6), crotonyl-CoA ($[M + H]^+$, *m/z* 836.3), 3-hydroxybutyryl-CoA ($[M + H]^+$, *m/z* 854.4), and the lithium salt of 3-hydroxybutyryl-CoA ($[M + Li]^+$, *m/z* 860.5).

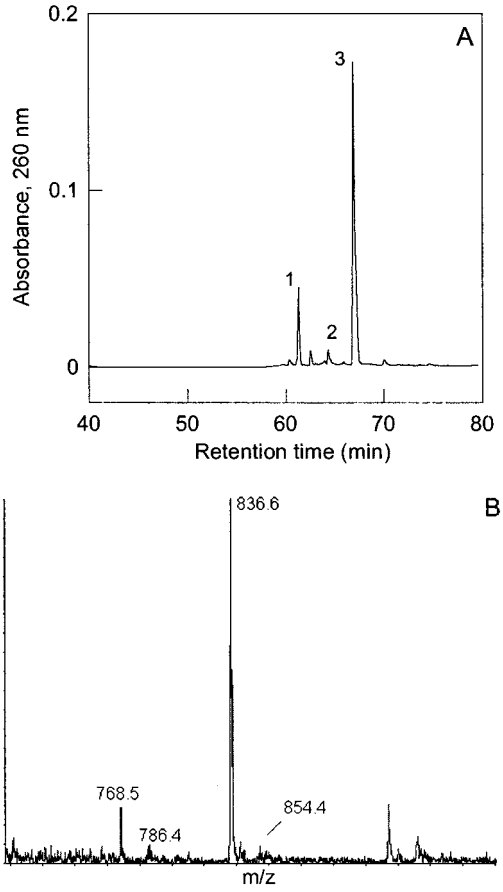

FIGURE 8: Identification of products generated by incubation of glutaconyl-CoA with Arg94Gln GCD. The mutant dehydrogenase, 10 μM, in 0.01 M ammonium formate, pH 7.6, 25 °C, was incubated with 28 μM glutaconyl-CoA for 10 min. The reaction was quenched by adjusting the pH to 3.0 with 1 M acetic acid, and the enzyme was removed by centrifugal filtration. (A) HPLC of the filtrate in solvent system B identified the compounds by retention times (in minutes): [1] *trans*-3-isomer of glutaconyl-CoA (61) and/or 3-hydroxybutyryl-CoA (62); [2] FAD (64); [3] crotonyl-CoA (67). (B) Mass spectrum of the analytes of the reaction mixture identified as coenzyme A ($[M + H]^+$, *m/z* 768.5), FAD ($[M + H]^+$, *m/z* 786.4), crotonyl-CoA ($[M + H]^+$, *m/z* 836.6), and 3-hydroxybutyryl-CoA ($[M + H]^+$, *m/z* 854.4).

consistent with the idea that glutaconyl-CoA is the enzyme-bound product of the reductive half-reaction.

3-Hydroxyglutaryl-CoA can be a product of the anaerobic reductive half-reaction of glutaryl-CoA with wild-type GCD. This product is generated by hydration of glutaconyl-CoA and its presence is not unexpected. *P. denitrificans* GCD (*7*), porcine medium chain acyl-CoA dehydrogenase (*21*), and *Megasphera elsdenii* short chain acyl-CoA dehydrogenase (*20*) also possess intrinsic enoyl-CoA hydratase activity, and activity was demonstrated with crotonyl-CoA. However, using optimal chain length substrates for the dehydrogenase reactions, the ratio of hydratase activity to dehydrogenase activity with the three enzymes are quite different. For human GCD with glutaconyl-CoA and glutaryl-CoA substrates, the ratio is 0.16. In the case of medium chain acyl-CoA dehydrogenase, 2-octenoyl-CoA is not hydrated. *M. elsdenii* short chain acyl-CoA dehydrogenases with crotonyl-CoA and butyryl-CoA substrates, the ratio is 0.0002. The active site cavities of medium chain acyl-CoA dehydrogenase and human GCD are relatively conserved between the two structures, and relatively nonpolar with the exception of Arg94 in GCD that replaces a Gln residue in medium chain acyl-CoA dehydrogenase (*28*, *29*). In contrast, the residues lining the cavity in the short chain dehydrogenase are somewhat less polar, and the cavity of the substrate binding pocket in the region of C-2 and C-3 of butyryl-CoA (and crotonyl-CoA) is much more constrained relative to medium chain acyl-CoA dehydrogenase and GCD due to changes in the E and G helices that form the binding cavity (*30*). Binding the preferred chain length substrate of medium chain acyl-CoA dehydrogenase desolvates the active site, and desolvation is less pronounced in the case of shorter chain length substrates which accounts for the hydration of crotonyl-CoA and the inability to hydrate 2-octenoyl-CoA. Steric exclusion of water in the region of C-2 and C-3 apparently limits the hydratase of the bacterial short chain acyl-CoA dehydrogenase. Therefore, it seems that a water molecule must remain near, or reenter, the reactive center of GCD in the presence of the product derived from the optimum substrate. The hydratase activity of GCD is dependent on Glu370, which functions as the catalytic base in the glutaryl-CoA dehydro-

genation pathway (*5*). Glu370 of GCD occupies a position, relative to an acyl-CoA substrate, that is very similar to Glu164 in crotonase. Glu164 apparently functions as the protic amino acid following addition of hydroxyl to crotonyl-CoA at C-3. In the hydration reaction Glu144 activates the water molecule to attack C-3 of the substrate (*31*−*33*).

The decarboxylation catalyzed by the oxidized enzyme seemingly contradicts the earlier experiments of Gomes et al. (*6*). In their experiments, *P. fluorescens* GCD was reduced aerobically with substrate, and decarboxylation of [1,5-^{14}C]-glutaryl-CoA was measured in the absence of an exogenous electron acceptor. In our experiments, glutaconyl-CoA was added aerobically to the oxidized Glu370Gln mutant enzyme, which is unable to catalyze abstraction of the α-proton and reprotonation of C-4 of glutaryl-CoA. At 5 s and 60 min, the *P. fluorescens* enzyme had undergone 0.27% and 31% of a single turnover, respectively. It is likely that the low turnover of the bacterial enzyme reflected a low level of oxidase activity. Oxidation of the dihydroflavin anion of the dehydrogenase (*5*) may certainly contribute to rate enhancement of the decarboxylation reaction. However, it is clear that the oxidized enzyme has decarboxylase activity for which the driving force is likely the presence of the γ-carboxylate in a medium of low dielectric. The redox-independent decarboxylase activity is not confined to the mutant enzyme, Glu370Gln GCD. Wild-type GCD has decarboxylase activity, as does the Arg94Gln mutant. Hydratase activity complicates analysis of the conversion of glutaconyl-CoA to crotonyl-CoA by the wild-type protein. An important finding in the experiments with Glu370Gln GCD is the observation of a relatively stable, long-wavelength- (725 nm-) absorbing species. This species is very similar to those ligand/acyl-CoA dehydrogenase binary complexes that have been characterized as charge-transfer species between a delocalized anion of a nonoxidizable ligand and a relatively electron-deficient, oxidized flavin (*5*, *24*−*26*). We propose that the long-wavelength-absorbing species is a charge-transfer complex between the delocalized crotonyl-CoA anion and the oxidized flavin. It is reasonable that this intermediate is detected with the Glu370Gln GCD for the following reasons. First, there is no general acid catalyst, Glu370(H^+), to reprotonate the anion that is presumably slowly protonated by diffusion of solvent protons into the active site. Gomes et al. (*6*) estimated that in the turnover of the bacterial GCD about 1% of the protons at C-4 of crotonyl-CoA are derived from solvent. Second, the presence of Gln370 in the mutant, rather than unprotonated Glu370 in the wild type, in the active site is expected to render the anion more stable in the mutant than in the wild type. Comparison of the rates of anion formation and decay suggest that decarboxylation is rate-determining with Glu370Gln under these conditions rather than protonation of the anion.

Polarization of acyl-CoA ligands by hydrogen bonding of the carbonyl oxygen of the thioester is a common and essential feature of catalysis by acyl-CoA dehydrogenases (*28*, *30*, *34*−*37*, *43*) and crotonase (*38*−*42*). Polarization by the hydratase stabilizes partial positive charge at C-3, promoting attack by the water molecule on the enoyl-CoA (*38*−*42*). GCD has not been crystallized with an acyl-CoA ligand in the active site. However, glutaryl-CoA was modeled into the active site according to the medium-chain dehydrogenase/octanoyl-CoA complex (*28*, *29*). The model permits hydrogen bonding of the substrate carboxylate with Arg94, appropriate positioning of the catalytic base, and hydrogen bonding of the 2′-hydroxyl of the ribityl side chain of FAD and the amide backbone hydrogen of Glu370 with the carbonyl oxygen of glutaryl-CoA (*29*). Considering the similarity of the dehydrogenation reaction of short-chain, medium-chain, and glutaryl-CoA dehydrogenases and the rather high hydratase activity of GCD, glutaconyl-CoA appears highly polarized, and this polarization probably contributes to the hydration of glutaconyl-CoA. Decarboxylation reactions usually involve an anion and a mechanism that delocalizes accumulation of negative charge on an atom that is not particularly electronegative (*42*, *44*, *45*). The polarization of glutaconyl-CoA responsible for hydration may also be involved in its decarboxylation since polarization would delocalize the negative charge at C-4 of the crotonyl-CoA anion following decarboxylation. The polarization may also lower the pK_a of the crotonyl-CoA anion. The pK_a of acyl-CoA α-protons can be lowered by 9−13 pK units by polarization (*34*, *46*, *47*). Polarization affected through hydrogen bonding of the carbonyl oxygen of thioesters is proposed to be involved in the decarboxylations catalyzed by *E. coli* methylmalonyl-CoA decarboxylase and of glutaconyl-CoA by *A. fermentans* glutaconyl-CoA decarboxylase (*44*, *48*). It will be of interest to determine whether substrate polarization via the thioester carbonyl participates in the decarboxylation of glutaconyl-CoA.

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